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Neuropeptidomics: Improvements in Mass Spectrometry Imaging Analysis and Recent Advancements

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Abstract

Neuropeptides are an important class of endogenous peptides in the nervous system that regulate physiological functions such as feeding, glucose homeostasis, pain, memory, reproduction, and many others. In order to understand the functional role of neuropeptides in diseases or disorders, studies investigating their dysregulation in terms of changes in abundance and localization must be carried out. Because multiple neuropeptides are believed to play a functional role in each physiological process, techniques capable of globally profiling multiple neuropeptides simultaneously are desired. Mass spectrometry is well-suited for this goal due to its ability to perform untargeted measurements without prior comprehensive knowledge of the analytes of interest. Mass spectrometry imaging (MSI) is particularly useful because it has the capability to image a large variety of peptides in a single experiment without labeling. Like all analytical techniques, careful sample preparation is critical to successful MSI analysis. The first half of this review focuses on recent developments in MSI sample preparation and instrumentation for analyzing neuropeptides and other biomolecules in which the sample preparation technique may be directly applicable for neuropeptide analysis. The benefit offered by incorporating these techniques is shown as improvement in number of observable neuropeptides, enhanced signal to noise, increased spatial resolution, or a combination of these aspects. The second half of this review focuses on recent biological discoveries about neuropeptides resulting from these improvements in MSI analysis. The recent progress in neuropeptide detection and analysis methods, including incorporation of various tissue washes, matrices, instruments, ionization sources, and computation approaches combined with the advancements in understanding neuropeptide function in a variety of model organisms indicates potential for the utilization of MSI analysis of neuropeptides in clinical settings.

Keywords

mass spectrometry imaging; neuropeptide; peptide; peptidomics; MALDI; LESA; MSI

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1. Introduction

One of the earliest recordings of a peptide functioning as a transmitter was reported in invertebrates in 1975 [1]. Since then, the field of neuropeptide research and neuropeptidomics has advanced greatly. Neuropeptides are secreted by a variety of tissues from the endocrine and nervous system and act by binding to membrane receptors on target cells to stimulate a signaling cascade [2]. Functionally, neuropeptides regulate almost every physiological process, such as feeding, growth, metabolism, reproduction, and environmental adaptation. Invertebrates have continued to be heavily utilized to study neuropeptide synthesis and modulation activity due to their relatively simple nervous system compared to mammals. Due to the advancements in analytical tools in the last two decades, more mammalian neuropeptides have also been discovered and characterized. For example, vasopressin and oxytocin were observed to act as hormones that regulate diuresis and lactation in mammals and their biochemical mechanism involved brain signaling [3]. Due to the highly transient and dynamic nature of neuropeptides, the ideal analytical tool would be able to capture all three aspects simultaneously. Although such technique does not exist, mass spectrometry (MS) is a good candidate due to its ability to rapidly interrogate virtually all analytes in a sample in a single analysis, providing both quantitative and structural information.

Mass spectrometry imaging (MSI) is a powerful technique capable of simultaneously characterizing the spatial distribution of hundreds of peptides by detecting their mass-to-charge (*m/z*) ratio. Although it offers advantages over traditional imaging techniques, such as not requiring the use of antibodies and multiplexing, biological discoveries using MSI could be greatly facilitated by coupling MSI with other relatively simple biochemical visualization techniques (*i.e.*, histological staining) and also by strategically imaging a large set of consecutive tissue sections to visualize the 3-D distribution of an analyte. As instrumentation is developed to allow separation of molecules prior to MSI measurement, frequency of MSI usage is increasing. Efforts to obtain neuropeptide localization information using MSI have been highly fruitful due to recent developments in sample preparation and instrumentation. Because neuropeptides are rapidly degraded *in vivo* by catabolic enzymes once an organism is sacrificed, efforts must be taken to rapidly measure endogenous neuropeptides by MSI or to immediately preserve the tissue. This review addresses recent tissue preservation methods suitable for peptide analysis, as well as methods to release the peptides from their preserved state. Additionally, due to the soluble property of neuropeptides, every solvent that comes in contact with the tissue has the potential to delocalize neuropeptides, which can skew localization or abundance results. This review discusses recent studies that examine different solvent treatments/washes in order to increase the number of detectable neuropeptides or enhance detection of, unsurprisingly, cohorts of peptides with similar characteristics. A major contributing factor to the recent success in uncovering new neuropeptides is the advancements in MS sampling and ionization sources. Due to its commercialization and consistent performance, matrix-assisted laser desorption/ionization (MALDI) is a popular choice of ionization source by many researchers. For example, one key benefit of newer MALDI-time-of-flight (TOF) instruments is the drastically shortened measurement time due to hardware improvements. A

compilation of different washes, matrices, instrumentation and analysis parameters from recent MSI of neuropeptide studies is summarized in Table 1.

Researchers have recognized the therapeutic potential that neuropeptides hold; therefore, many studies have been carried out examining differences in neuropeptide distribution and relative signal intensity between tissues from a control state and a disease state. This is not a trivial task due to the inability to acquire true technical replicates from tissue section measurements and the inherent difficulty in capturing the exact z-plane section of the tissue for both control and disease state tissues. However, with the aid of external validation techniques, such as immunohistochemistry, many notable neuropeptide discoveries have stemmed from MSI techniques. Thus, the final portion of this review will discuss how MSI has advanced our understanding of neuropeptide function within a variety of model organisms, including humans. Studies that were focused on in this review were chosen if (1) they were published in 2017 or later (with a few notable exceptions that were foundational studies), (2) if the study involved MSI analysis of neuropeptides, and (3) involved MSI techniques that are directly applicable to neuropeptide research. We strived to cover publications that, at least, fell into the first two categories, although there may be some that were missed.

2. Foundations in Imaging: Basic Considerations of Imaging Commonly Applied to Neuropeptide Analysis

2.1 Tissue Washes

Sample preparation methodology has a large impact on successfully performing MSI measurements and maintaining reproducibility between measurements. For researchers interested in studying individual tissues, then, naturally, the first consideration will be related to the tissue dissection step. Placing animal tissues in a buffer facilitates dissections and is often necessary for dissections of small tissues. The buffer may play a large role in the final tissue structure and integrity, which will directly impact tissue section morphology. Buffers such as phosphate-buffered saline (PBS) or physiological saline are commonly used during tissue dissection prior to imaging because they are assumed to have a neutral effect on tissue morphology due to its pH buffering activity. However, it is plausible that neuropeptide detection may be enhanced by optimization of dissection buffer. A logical first step would be to use buffers with low salt concentrations as salt has been shown to cause ion suppression effects and degrade MALDI-MS spectra [4]. To the best of our knowledge, no dissection buffers have been recognized to enhance neuropeptide detection; however, optimization of dissection buffers for improved detection of other analytes for MS imaging have been reported [5]. In a study by Yang *et al.*, they found that using a dissection buffer with a low salt concentration (50 mM ammonium bicarbonate) caused tissue hardening and breakage, which was likely due to excessive dehydration despite the buffer being at an isotonic salt concentration [5]. The use of PBS buffer, which has a much higher salt concentration, did not affect tissue morphology but caused delocalization of lipids. They also bring up an alternative method to bypass dissection buffer effects by not dissecting the tissue out of the animal at all, but to cryosection the whole body of the animal. Of course, this greatly increases experimental time because as many as 100 sections must be imaged to acquire

comprehensive information about the tissue, assuming that the tissue can be visualized during cryosectioning at all, whereas dissection of the tissue and directly placing it on a glass slide will result in a single measurement. Another study briefly compared the effect of dipping the tissue in saline and saline with 33% glycerol immediately post dissection, and both approaches showed similar results in neuropeptides detected [6]. Although the authors did not discuss the rationale for adding glycerol, a potential explanation is gently preserving the tissue by applying glycerol in low concentrations [7]. For crustacean neuropeptide imaging studies, our lab dissects tissues in physiological saline followed by quickly dipping the tissue in deionized water immediately prior to embedding the tissue in order to reduce the salt content without compromising the integrity of the tissue [8]. This theme of a trade-off between analyte signal enhancement while minimizing delocalization is carried throughout the entire sample preparation workflow.

A major challenge in analyzing neuropeptides by MSI is signal masking by more abundant biomolecules, most likely due to a lack of separation prior to MS analysis, which provides partial explanation for why fewer neuropeptides are observed from MSI measurements than from tissue extract measurements. Brain tissue, for example, is commonly utilized for neuropeptide analysis because many neuropeptides are synthesized throughout the brain [9]. However, the brain not only contains region-specific endogenous neuropeptides but also hosts a large variety of abundant lipids [10]. Essentially, to remove masking species such as lipids, several groups utilized solvents that are optimized for extracting lipids to remove them from the tissue sample prior to peptide analysis. For example, Matsushita *et al.* reported that using supercritical fluid of CO_2 to wash the tissue sample for 1 hour enhanced peptide signal compared to unwashed mouse brain tissue [11]. The rationale is that combining the characteristics of a supercritical fluid (low viscosity and high diffusivity) with an organic solvent will allow the solvent to deeply penetrate the tissue and enhance its ability in removing lipids from a tissue. An added benefit of this method is that CO_2 is nontoxic. This method increased the signal of peptides in the *m/z* 3500–5000 mass range up to 190-fold higher than the control (unwashed) without changing the morphology of the tissue section. Ong *et al.* also incorporated supercritical CO_2 fluid in their sample preparation workflow by rinsing the tissue section using ice-chilled 150 mM ammonium acetate for 10 s, immediately followed by isopropyl alcohol for 30 s, and dried the tissue section by supercritical drying [12]. The wash removed signals that interfered with peptide signals and the supercritical drying produced less cracks in the tissue than drying by N_2 gas or using a desiccator. Many other groups have developed wash protocols to gently remove salts and lipids from the tissue. Maslov *et al.* conducted a thorough investigation of 17 different wash protocols on cured ham tissues to find a wash that decreased lipid signal and increased peptide signal without creating matrix “sweet spots”, or locations with high concentrations of matrix crystals [13]. They found the optimal wash protocol to consist of subsequent washes of 100% isopropanol @ 60 s, 70% isopropanol @ 60 s, Carnoy’s solution (C.s.) @ 120 s, 70% isopropanol @ 60 s, modified C.s. (60 % (v/v) ethanol, 30 % (v/v) methyl-tert-butyl ether, 10 % (v/v) glacial acetic acid) @ 120 s, 100% isopropanol @ 60 s, ultrapure water @ 240 s, 70% isopropanol @ 60 s, and 100% isopropanol @ 60 s. Another finding from the same study was that repetition of several washing steps resulted in cross-contamination with embedding medium (this was not observed in the optimal wash

protocol), which was validated with light microscopy. Thus, we recommend to first determine whether or not an embedding medium is actually needed for successfully sectioning a tissue of interest. Additionally, they note that the ethanol-based washes they tested did not improve signal quality in the *m/z* 1000–4000 range, while other groups studying proteins in higher mass ranges did notice an improvement in signal quality using ethanol-based washes [14, 15]. This discrepancy may be due to ethanol's ability to reduce inorganic salts, and the ion suppression effect of inorganic salts may not evenly act on all peptides. A recent study from Buchberger *et al.* also investigated ethanol-based washes and reported that submerging a blue crab brain tissue section in 50% ethanol for 10 seconds increased signal intensity of 34 neuropeptides while signal from 6 neuropeptides were decreased [16]. Neuropeptides that were enhanced by this wash were overall less hydrophobic than the neuropeptides whose signals were decreased, and all of them contained at least one arginine residue (Figure 1). Another study investigating endogenous peptides in the *m/z* 900–2000 range comes from Potočnik *et al.*, where they dipped rat pituitary gland tissue sections into three consecutive washes: ice-cold $CHCl_3$, MeOH, and EtOH for 30 s, 30 s, and 1 min, respectively, prior to applying matrix [17]. Another variation of ethanol-based washes comes from Sui *et al.*, where they washed spinal cord tissue sections with 70% ethanol for 10 s, followed by 95% ethanol twice for 10 s each prior to MSI analysis for neuropeptides in the *m/z* 1000 – 1400 range [18]. Chloroform was also employed to increase the number of observable neuropeptides in mammals without causing delocalization, which was verified with neuropeptide standards spotted on the tissue section prior to the chloroform application [19]. It is worth noting that almost all of these groups dry their tissue sections, usually with a vacuum desiccator, before and/or after applying a wash and before spraying matrix in order to minimize analyte delocalization.

2.2 Preserved Tissues

Each animal model and tissue type presents unique advantages and challenges for neuropeptide studies. Most of the studies in this review utilize tissue from mice or invertebrates (crustaceans, cockroaches, *etc.*). For studies heavily focused on instrument evaluation, the choice of animal model has a smaller impact than the choice of specific neuropeptide targets to be used as a benchmark for successfully reaching their goals. For studies mainly focused on elucidating neuropeptide function, the choice of animal model is more critical for the success of the studies. If the goal is to perform an untargeted neuropeptide study that will potentially encompass homologous peptides, invertebrates are a good choice due to their well-characterized nervous system, as well as the fact that many peptidergic signaling pathways and general principles of neuromodulation are conserved between crustaceans and mammals, thus the lessons learned can be translated to the mammalian system. However, certain biological questions require the direct analysis of mammalian tissue, and the most common form of preserved tissue is formalin-fixed paraffin embedded (FFPE) tissue. Recently, studies have emerged that show successful detection of endogenous peptides from FFPE tissue. In the study from Reglodi *et al.*, deparaffinization occurred by applying two changes of xylene followed by rehydration in isopropanol and ethanol [20]. Antigen retrieval was subsequently performed using a decloaking chamber prior to trypsin digestion and matrix deposition to de-crosslink peptides, enabling detection of several peptides in the *m/z* 1000–2000 range. Paine *et al.* took a different approach by

developing a modified deparaffinization method consisting of xylene (100% @ 3 min), fresh xylene (100% @ 2 min), ethanol (100% @ 2 min), and fresh ethanol (100% @ 1 min), with antigen retrieval omitted completely [21]. Background subtraction was applied on the obtained mass spectra, yielding more than 20 peptide signals with high signal-to-noise in the *m/z* 500−2000 range. Because antigen retrieval was not performed, one can assume that these peptides contained characteristics that allowed them to escape formaldehyde crosslinking. These peptides were short (< 20 residues), did not contain lysine residues, and a significant number of them contained a blocked N-terminus. While the original goal of this study was to make FFPE tissues accessible for imaging of endogenous peptides, which was met, it also established precedence for development of additional methods for chemically modifying the tissue to aid in detection of endogenous peptides. More recently, the same research group conducted a parallel study evaluating the effect of performing formalin fixation on fresh-frozen tissue prior to MSI analysis [22]. Although this study did not focus on peptides, they found that applying formalin resulted in depletion of amine-containing lipids with minimal delocalization effects. Combined together, these findings potentially provide a basis for identifying ways to chemically modify interfering biomolecules (*i.e.*, lipids) and render them unable to be detected during MS measurements, thus minimizing their ability to mask neuropeptide signal.

Another method for enhancing detection of endogenous peptides is tissue preservation. In MS analysis where neuropeptides are extracted from tissue homogenate, these workflows often involve inactivating proteases that would otherwise generate abundant protein fragments (false positives) and mask neuropeptide signal (false negatives) [23]. Examples include dissection immediately followed by placing the tissue in boiling water or acidified methanol to inactivate or precipitate proteases [24]. Efforts to translate this process to MSI workflows have involved heat preservation while maintaining morphology. One of the first examples is a report from Goodwin *et al.* demonstrating that mounting mouse brain tissue sections on conductive carbon tape prior to applying the snap heat stabilization device Stabilizor T1 (Denator AB, Uppsala, Sweden) did not alter morphology for neuropeptide analysis by MSI, whereas non-heat treated tissues showed peptide degradation products [25]. Another example of the use of the Stabilizor T1 is from Griffith *et al.* where they compared the protein profile of mouse tissues treated for 45 seconds at 95 °C immediately after dissection to tissues that were frozen in isopentane [26]. Here, they saw a 1.5-fold improvement in the number of proteins (4–16 kDa) detected when heat-treated tissue was compared with frozen tissue. Although the study from Griffith *et al.* examines proteins larger than many neuropeptides, their findings show that heat-preserved tissue does not change peptide localization compared to frozen tissue.

2.3 MALDI-MS Matrix and Instrumentation

Currently, MALDI has been the main ionization source for MSI due to its prevalence in qualitative and, recently, more quantitative research of large biomolecules such as peptides/proteins, carbohydrates, nucleotides, lipids, and many other classes of molecules contained in complex biological samples, which is a direct result of the soft ionization offered by the matrix. One of the final considerations made for sample preparation for MALDI-MS analysis is choice of matrix. Table 1 shows that 2,5-dihydroxybenzoic acid (DHB) is

commonly used for neuropeptide analysis. Sui *et al.* tested different ammonium salt compositions and amounts of DHB to see which system would produce the highest neuropeptide signal and lowest heme signal in mouse spinal cord tissue [18]. The optimal matrix system was found to be 25 mg/mL DHB in 50% MeOH with 10% ammonium acetate and 3% trifluoroacetic acid (TFA). This strategy of choosing a matrix system that enhances detection of peptides while simultaneously suppressing interfering species is of high interest and should be applied whenever possible, especially when there are distinct characteristics between the two. In regards to determining appropriate matrix concentration, Smolira *et al.* found that choosing a concentration that is too low will produce a bias and only allow biomolecules with higher affinities for charge than others to be ionized, while a concentration that is too high will lead to high interference from numerous biomolecules [27]. Although this MALDI analysis was applied for peptide extract or peptide standard and not for imaging of tissue, this general strategy may be applied to future imaging studies. Hulme *et al.* employed a unique matrix strategy where some brain tissues were treated with a highly efficient neuropeptide extraction matrix, but formed large matrix crystals which resulted in a lower spatial resolution (50 μm), whereas some brain tissues were treated with a less efficient extraction matrix, but allowed higher spatial resolution (25 μm in primate and 15 μm in rat brain), in order to gain the most comprehensive neuropeptide information [19]. The same group also developed pyrylium salts as matrices that can derivatize neuropeptides by reacting with primary amine functional groups, which can improve sensitivity for neuropeptide detection by MALDI-MS imaging [28]. It is our opinion that it is unlikely that all neuropeptides may be grouped together for a single matrix system, but more likely certain matrix systems will work well for a group of peptides with similar masses and hydrophobic/hydrophilic character, and the tissue type will also play an important role in matrix system penetration and crystal formation.

Although MALDI typically operates under vacuum, recent studies reported on the benefits of atmospheric pressure (AP) MALDI for peptide detection. For example, Chen *et al.* demonstrated that coupling the AP/MALDI (ng) UHR ion source (MassTech, Columbia, MD, USA) with an Orbitrap MS instrument allowed faster acquisition speed (7x faster) and higher spatial resolution (40 μm) than a commercial vacuum MALDI-Orbitrap instrument [29]. Using this system, they successfully detected neuropeptides from crustaceans and rats at comparable sensitivity despite softer ionization than vacuum MALDI. Areas of potential future development are to evaluate detection of volatile peptides as well as expand matrix options to include those that were previously too volatile to be used under vacuum conditions. A more recent study from Li and coworkers expanded on these studies and investigated the utility of a subatmospheric (subAP)-MALDI ionization source (MassTech, Columbia, MD, USA) for MSI analysis of neuropeptides [30, 31]. Notable results stem from their comparison of different raster sizes where they showed that a laser spot size of 50 μm resulted in a greater number of neuropeptide identifications than 25 μm, likely due to a sensitivity issue in which a smaller spot size results in a fewer number of ions being introduced into the mass spectrometer. Additionally, using ITO-coated slides was also superior to using regular glass slides. Overall, the subAP ionization source offers improvements in performance and ion collection after optimized intermediate pressure conditions are applied to lessen the ion suppression effects as discussed in the publication.

The relative ease of switching between an electrospray ionization (ESI) and MALDI ionization source on Thermo Orbitrap instruments is an added benefit. To further increase the number of observable neuropeptides, DeLaney *et al.* took a unique approach in combining MALDI with capillary electrophoresis (CE) for the separation of neuropeptides producing a trace which was analyzed by MSI [32]. Although this was an MSI measurement, the main goal was to improve neuropeptidome coverage rather than spatial localization of neuropeptides in tissue samples. By utilizing larger injection volumes and enhanced separation resolution with a positively-charged polyethyleneimine (PEI) capillary coating that reduced peptide adsorption and reversed electroosmotic flow (EOF), the number of neuropeptides detected doubled compared to a conventional MALDI spot analysis of neuropeptide extract.

2.4 Other Ionization Sources

Other ionization sources have been used for detecting neuropeptides. Potočnik *et al.* demonstrated the first application of matrix-enhanced secondary ion mass spectrometry (ME-SIMS) for on tissue *de novo* sequencing of neuropeptides from rat pituitary gland at higher spatial resolution (1.2 μm raster size) than MALDI-MS [17]. The rationale is that the matrix offers softer ionization for reduced primary source fragmentation, as well as increased ionization efficiency, over SIMS alone. In a study by Griffith *et al.*, the peptide profiles of rat tissues from liquid extraction surface analysis (LESA) MSI and LESA multistep high field asymmetric waveform ion mobility spectrometry (FAIMS) MSI measurements were compared [26]. The addition of FAIMS to the instrumentation showed an improvement in the number of proteins identified. Here, 40:60 acetonitrile (ACN):water with the addition of 1% formic acid (FA) was used to sample and extract peptides from discrete points on tissue sections and the extract was delivered into the MS instrument by the Triversa Nanomate chip-based electrospray device. The spatial resolution of LESA MSI was improved by Lamont *et al.* from 2 mm to 0.4 mm by coupling LESA with a μLC system containing a silica capillary, which allowed the confident identification of neuropeptides from rat pituitary glands [33]. Here, the extraction solvent used was ACN/water/formic acid, 70/30/0.1 v/v/v, and it is worth noting that when choosing a solvent, one must also consider the stationary phase of the column as this choice will impact types of species extracted.

Due to the difficulty in obtaining MS/MS spectra, accurate mass matching is a standard method of identifying neuropeptides for MSI. A common next step is to verify the presence of specific neuropeptides by homogenizing the tissue and analyzing the neuropeptide extract with LC-MS/MS, usually using a high mass resolution instrument [20, 34, 35]. Useful additional verification methods include performing laser microdissection to isolate and extract neuropeptides from specific regions of tissues, and further analyzing the identified peptides by generating antibodies for immunohistochemistry [20]. Although MALDI-TOF instruments provide many advantages, such as fast MSI acquisition speed and higher mass range, it is often insufficient to rely on accurate mass matching alone for verification, and on-tissue MS/MS is notoriously challenging. A recent paper by Yang *et al.* investigated lipids by MSI and achieved less than 40 ppm error by using known lipid signals from liver homogenates to obtain five points of calibration over the considered mass range [5]. Table 1 lists the calibrants used in recent MALDI-TOF neuropeptide MSI studies, and due to the

heavy usage of MALDI-TOF instruments, it would be of high interest to develop this area and improve calibrant choices for neuropeptides. It is important to note that optimization of instrument parameters by comparing different conditions is not a trivial task. At the very least, applying statistical tests (*i.e.*, t-test) to calculate the effect of each parameter should be carried out to maintain rigor and reproducibility [35].

3. Neuropeptide function

Once a neuropeptide has been identified *in situ* and its sequence confirmed, its function is interrogated for biological activity and potential therapeutic utility. Functional studies typically involve dosing and behavioral studies, as well as electrophysiology, tissue-specific assays, and various optogenetic tools. Additionally, quantitative MS has been useful for large-scale profiling of differentiation between control and disease or treated states. This method is useful for getting a broad picture of how the relative abundance of neuropeptides differ. Recently, MS imaging has demonstrated substantial utility in assisting in the functional assessment of neuropeptides. It is particularly advantageous because it is non-targeted, enabling simultaneous localization of multiple neuropeptides, which facilitates the understanding of neuropeptide colocalization and comodulation. Although MS imaging alone cannot provide specific functional information about individual neuropeptides, the information gained enables global trends to be observed and facilitates follow-up functional characterization with more targeted methods. Furthermore, colocalization of multiple neuropeptides in an anatomical structure may suggest functional interaction of these signaling molecules and regulatory pathways. In the following section, we highlight some exciting advances that have been made toward improving the understanding of neuropeptide function. While the studies highlighted do not provide a comprehensive catalog of the field, they indicate some notable recent advances that are indicative of where the field may be heading.

3.1 Invertebrate model organisms

Numerous model organisms have been investigated for insight into the mechanisms behind neuropeptide-altered disease states and how these states are altered by various drug treatment tests. The organisms investigated for functional analysis range in complexity from simple invertebrates containing few, easily-distinguishable neurons, to complex mammalian systems including humans. Simplified model organisms are useful for obtaining fundamental information about how neuropeptides impact circuit dynamics and how changes in environmental and physiological conditions impact neuropeptide expression. For example, the Sweedler group takes advantage of the well-characterized neuropeptide profile of *Aplysia californica* to implement novel imaging methods for studying cell transport. Using a method of stretching tissue across a membrane, detailed characterization of neuropeptides within individual neurons were able to be obtained with MSI [36–39]. The method is particularly of value for studying neurons cultured under different physiochemical conditions, and can be applied to studying neurons belonging to neural circuits in more complex model organisms. Additionally, the Sweedler Lab implemented MSI for accurate, label-free quantitation of neuropeptide release from single cells using a novel microfluidic device [40, 41]. The device includes a channel coated in a synthetic substrate that binds to

neuropeptides as they are released from the cell, which can be visualized by MALDI MSI. Consequently, the abundance of a neuropeptide can be determined by measuring the length of channel containing bound neuropeptide, rather than relying on the less-accurate peak areas obtained from imaging experiments. The method provides valuable information about the function of neuropeptides within individual neurons by measuring magnitude of neuropeptide release triggered by various stimuli.

Invertebrate model organisms have been extensively utilized in neuropeptide research, and much attention has been given to functional assessment [42]. The parasitic worm, *Ascaris suum*, is particularly advantageous because it possesses a small number of neurons, reducing the complexity of both the neuropeptides and their mode of action. By isolating each nerve structure, *e.g.*, nerve ring, ventral ganglion, and dorsal ganglion, and mounting them on a MALDI target plate, the individual neuropeptide composition of each structure was able to be profiled [43]. By combining multiple MALDI MS platforms with direct analysis, a total of 39 neuropeptides were mapped, including 17 neuropeptides reported for the first time. Interestingly, the researchers found that each nerve structure possesses a unique peptide expression profile, providing preliminary insight into the likely function of the various neuropeptides and highlighting key neuropeptides for downstream functional studies.

Another invertebrate model organism that has been invaluable to neuropeptide research is the decapod crustacean *Cancer borealis*, as well as other crab species such as *Callinectes sapidus.* Crabs are particularly useful for electrophysiological studies because their nervous system is comprised of several well-defined neuronal circuits with relatively large and easily-distinguishable neurons. Much research has been dedicated to localizing neuropeptides to specific areas of the nervous system, enabling improved understanding of neuropeptide modulation within the various circuits. Specific neural tissues within the crustacean nervous system include the stomatogastric ganglion [44], brain [45], pericardial organs [45], and the entire stomatogastric nervous system [44]. The results of mapping neuropeptides in these various tissues reveal a functional relationship between neuropeptide isoforms belonging to the same family. Three dimensional distributions of neuropeptides within the brain have also been constructed by acquiring MS images of consecutive brain sections and compiling them into 3D maps using software tools, thus providing even deeper insight into the specific localization of signaling peptides within the brain tissue [46]. Furthermore, differences in biological conditions have also been compared in crustacean tissue using MSI in order to explore the relationship between neuropeptides and specific phenotypes. The differences in localization due to feeding have been studied in both brain and pericardial organ tissue [34, 47]. Additionally, the differences in neuropeptide expression due to environmental stressors, namely hypoxia and hypercapnia, have also been explored [16]. Differences in localization and intensity of some neuropeptides between control and experimental states indicate potential function related to the underlying biological mechanisms.

Insects represent a series of particularly useful invertebrate model systems for studying the relationship between neuropeptides and behavior outputs due to the well-characterized, discrete regions of insect brains. The most widespread insect model is fruitfly *Drosophila melanogaster*, whose genome was fully sequenced in 2000 [48]. Since then, this species has

been used to spatially profile numerous neuropeptides with MSI [49–51]. In one study that mapped neuropeptides in the brain of *D. melanogaster* sections, it was found that tachykinin and allatostatin neuropeptides are localized to distinct regions of the brain, and short neuropeptide F-like peptides were localized to the mushroom body of the brain, an area associated with memory and learning [51]. The African honeybee has also been the subject of neuropeptide characterization with MSI, resulting in interesting findings related to behavior. One notable example is differential expression of neuropeptides in regions of the brain during aggressive behavior. Allatostatin and tachykinin peptides, previously with unknown roles related to aggression, were found to act in different neuropils of the brain, indicating a potential role in neuromodulation related to this behavior [52]. Some representative MALDI-MS images are shown in Figure 2, and the results were confirmed with behavioral assays. Another study using African honeybees found that tachykinin and allatostatin-family neuropeptides are also differentially localized at different developmental stages that correlate with specific behavioral activities (*e.g.*, brood capping at young ages and foraging at older ages) [53]. MALDI-MSI has also been used to localize neuropeptides in other insect species, such as grasshopper [54] and cockroach [35, 55], and it is likely that more functional-driven studies on these species will follow.

3.2 Mammalian Organisms

While model organisms with simplified nervous systems are valuable for understanding the basic mechanisms of neuromodulations, mammalian models offer a closer comparison to humans that is useful for development of disease diagnosis and therapeutics. Some exciting progress has been made recently both to profile the localization of neuropeptides within mammalian brain tissue and compare differences in these localizations due to various conditions. The results of this study provide an improved understanding of the function of habenular nuclei in brain signaling. A method was recently developed for applying MALDI-MSI to study pain-related neuropeptides in the rat spinal cord [18]. The method was able to localize pain-related neuropeptides belonging to three families, originating from prodynorphin, proenkephalin, and protachykinin-1 proteins. Each neuropeptide family was found to have a distinct localization pattern. These findings will facilitate future applications for understanding pain-related diseases. Additional studies have examined neuropeptides related to other disease-related conditions, such as opioid peptides involved in Parkinson's disease in rats [56] and neuropeptides differentially expressed in obese mouse models [57]. A particularly notable study examining neuropeptide localization related to Parkinson's disease was recently published by the Andrén Lab, in which a high-resolution MALDI-MSI method was developed to characterize changes in neuropeptide localization in brains of rat models with and without L-DOPA treatment [19]. Reproducible images were obtained for over 20 neuropeptides, including dynorphins, enkephalins, tachykinins, and neurotensin. Human samples have also been interrogated for neuropeptide localization, as was done for localizing Aβ peptide deposits in the brain of Alzheimer's disease patients, showing that some Aβ peptide variants were localized to leptomeningeal vessels and others were localized to the cerebral parenchyma as senile plaque [58]. These results show potential for using MSI techniques for future clinical analysis of neuropeptides and facilitate the development of improved disease therapeutics.

3.3 Multimodal imaging and software techniques

While MSI is a powerful technique in neuropeptide research, it is often not sufficient to answer a biological question on its own. When this is the case, it is often helpful to combine MSI with other imaging techniques, most commonly histology. For example, Reglodi *et al.* were interested in observing the role of pituitary adenylate cyclase-activating polypeptide (PACAP) in accelerated pre-senile systemic amyloidosis. They used histopathology to identify age-related systemic amyloidosis in various tissues, and MSI revealed that apolipoprotein A-IV and several other proteins were present in the deposits [20]. Histology and MS images are shown in Figure 3, demonstrating the complementary information obtained from each imaging technique. Complementary modalities have been used for a variety of other recent applications, such as profiling peptides and lipids in the human primary visual cortex [59] and studying the bioconversion of dynorphin neuropeptide in various regions of a rat model [60]. By combining different imaging modalities, more detailed information can be obtained related to the functional role of neuropeptides within tissue. However, as neuropeptides elicit a signaling cascade by binding to G- protein coupled receptors (GPCRs), follow-up studies are necessary after imaging experiments in which receptors are identified and localized with knockdown or knockout neuropeptide or receptor genes. The data obtained from a large-scale MSI experiment can provide the first step that leads to downstream hypothesis-driven tests that enable in-depth functional analysis of individual neuropeptides.

4. Conclusion and Future Perspectives

Neuropeptide research has been greatly assisted by the recent developments in MSI. With the advantages of simple sample preparation and untargeted analysis, discrete localization patterns have been able to be mapped for a wide range of neuropeptides in numerous organisms, tissues, and disease states. While there are still limitations in the detection power of MSI, these issues are gradually being overcome by advancements in sample preparation protocols and ionization methods. Optimized wash protocols have been demonstrated to be promising for reducing signal interference from other species, most notably lipids, though care still needs to be taken to avoid analyte delocalization. Attention has also been directed to enhancing neuropeptide signal through stabilization procedures and steps to improve ionization. More progress is expected to be made in instrumentation and software analysis in order to further improve ionization and enhance the confidence of identifications. Currently, a limitation of MSI is the difficulty in obtaining unambiguous identifications. This challenge is being steadily overcome by utilizing high-resolution MS for improved mass accuracy and additional validation methods through follow-up analyses such as LC-MS/MS and immunohistology. While on-tissue MS/MS is currently still a difficult task, the additional information gained drastically improves the confidence of neuropeptide identifications, so it is likely that additional improvements will be made in this area as well.

The optimized imaging methods have been applied across a variety of organisms to better understand the function of these neuropeptides, from basic research in invertebrates, to complex disease model studies involving mammals. It is expected that we will see many more large-scale studies investigating neuropeptide localization, particularly in relation to

specific diseases. The results of these studies will likely facilitate the development of therapeutics to treat neurological disorders, and we can expect MSI to eventually find its place in the clinical setting to map neuropeptides in patient samples.

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List of abbreviations:

°C	degrees Celsius
ACN	acetonitrile
AmAc	ammonium acetate
AP	Atmospheric pressure
C.s.	Carnoy's solution
CE	capillary electrophoresis
CHCA	α–cyano–4–hydroxycinnamic acid
$CHCl_3$	chloroform
CO_2	carbon dioxide
DAN	1,5-diaminonaphthalene
DHB	2,5-dihydroxybenzoic acid
EOF	electroosmotic flow
ESI	electrospray ionization
EtOH	ethanol
FA	formic acid
FAIMS	field asymmetric ion mobility spectrometry
FFPE	formalin-fixed paraffin embedded
GPCR	G-coupled protein receptor
IPA	isopropanol
kDa	kilodaltons

KO	knock out
LC	liquid chromatography
LESA	liquid extraction surface analysis
m/z	mass to charge
MALDI	matrix–assisted laser desorption/ionization
MeOH	methanol
ME-SIMS	matrix–enhanced secondary ion mass spectrometry
min	minute
mm	millimeter
MS	mass spectrometry
MSI	mass spectrometry imaging
NP	neuropeptide
Ng	next generation
PACAP	pituitary adenylate cyclase-activating polypeptide
PBS	phosphate-buffered saline
PEI	polyethyleneimine
s	second
$scCO_2$	supercritical carbon dioxide
TFA	trifluoroacetic acid
TOF	time-of-flight
um	micrometer
v/v	volume per volume

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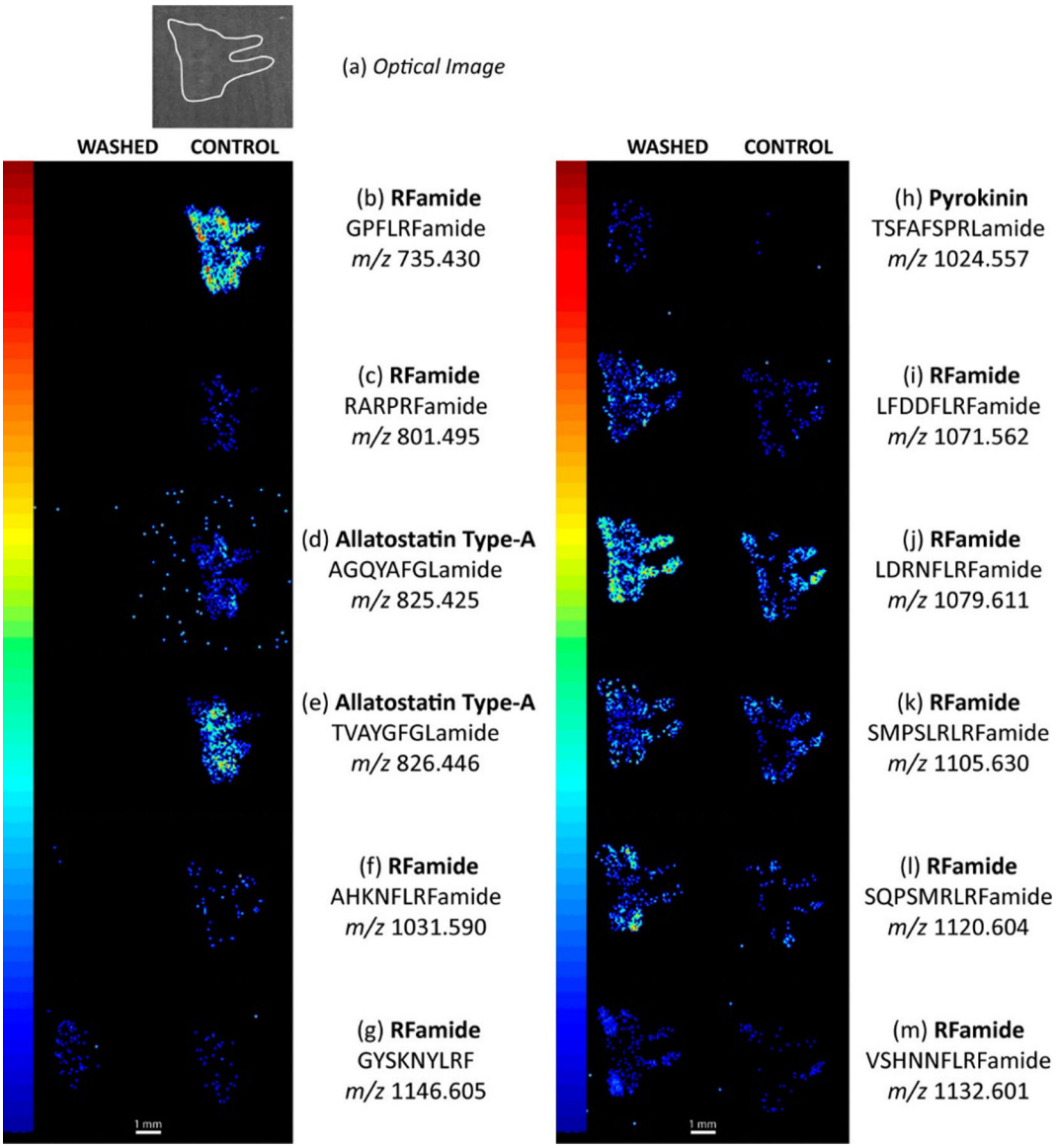

Figure 1.

MALDI-MS images of neuropeptides from crustacean brain. Washed sections were washed with 50% EtOH for 10 seconds. (a) An optical image of the crustacean brain is outlined in white. (b)–(f) Crustacean neuropeptides that were removed due to the washing step. (g) Neuropeptide image example that did not have a change in intensity or localization due to the washing step. (h)–(m) Neuropeptides that had a clear signal increase due to the washing step. Used with permission [16].

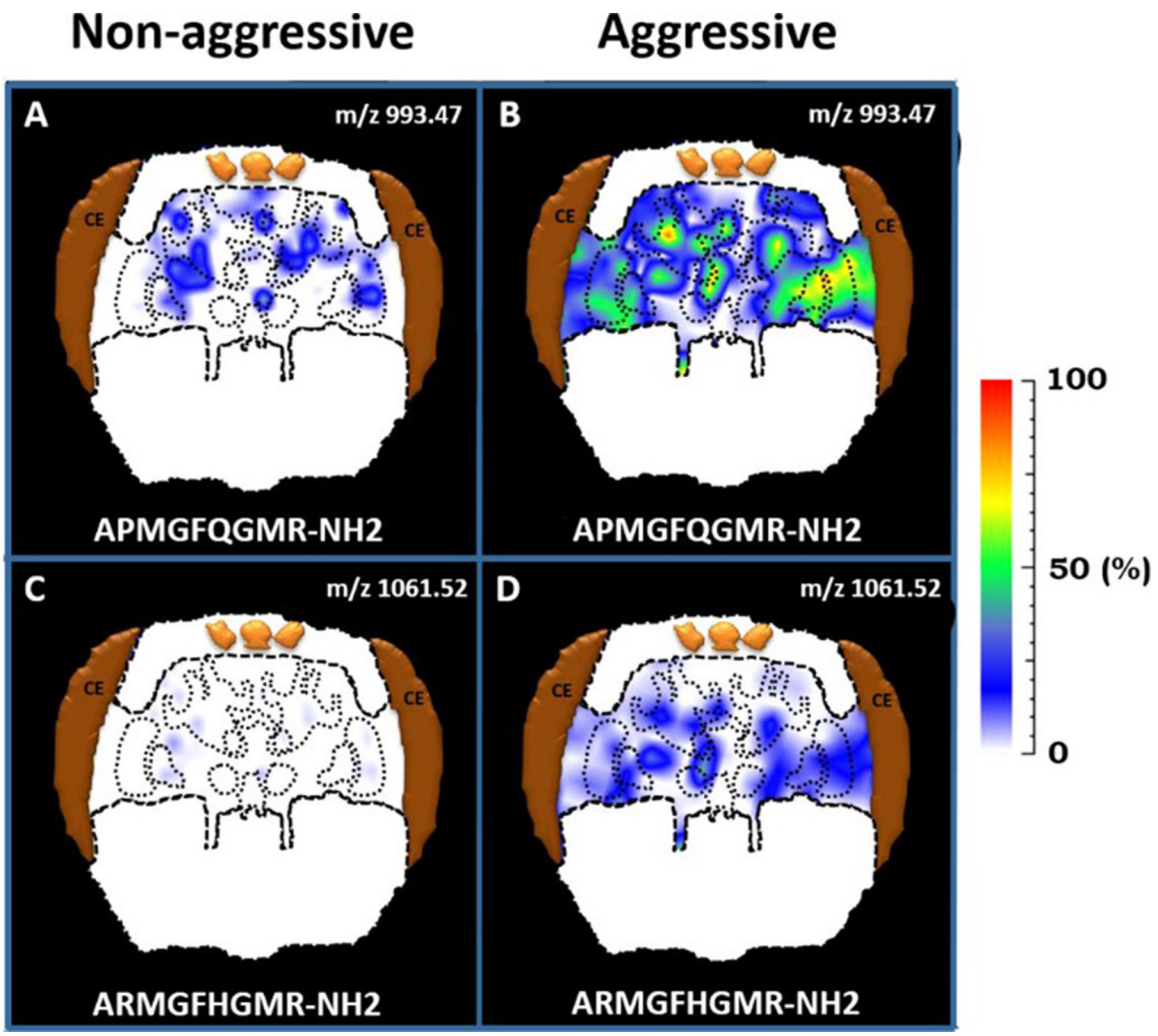

Figure 2.
MALDI-MS images of two mature tachykinin neuropeptides, APMGFQGMR-NH2 (A, B) and ARMGFHGMR-NH2 (C, D), showing differences in spatial distribution in the brain between non-aggressive (A, C) and aggressive (B, D) honeybees. Used with permission [52].

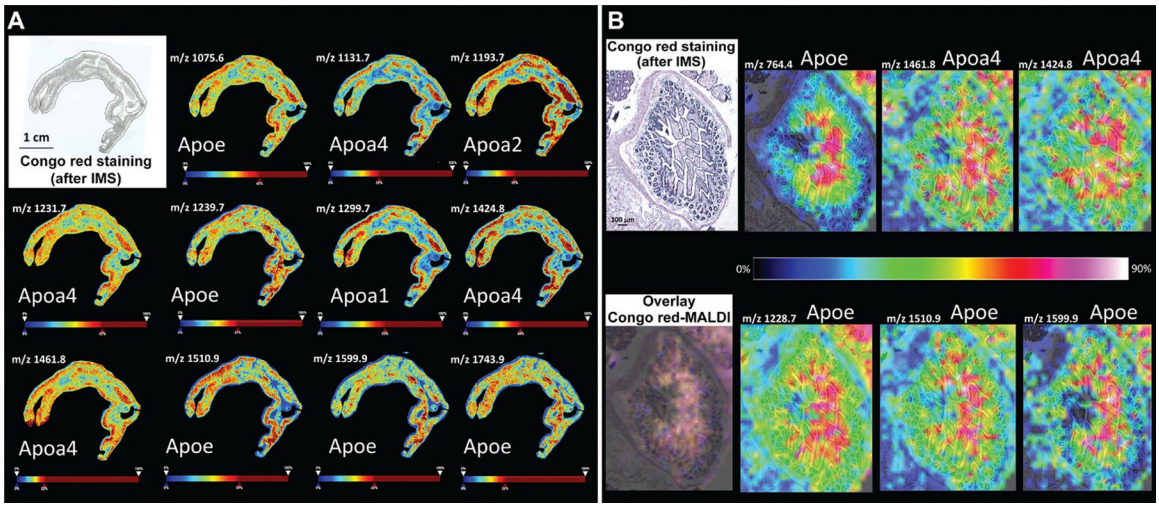

Figure 3.

Images of mouse intestine tissue sample, showing the correlation between Congo red-stained tissue and MALDI MS images for Apoe peptides. The Congo red stain correlates with the distribution of Apoe peptides (A), and co-localization of various *m/z* values correlates well with the amyloid deposits (B). Used with permission [20].

Table 1.

Sample preparation and instrumentation of MSI analysis of neuropeptides (NP) from selected recent publications.

Ref.	Tissue Type	Wash Steps	Matrix	Instrument	Calibration Samples	Analysis Software	Summary of Results
[11]	C57BL/6JJcl 8-week-old female mouse brain	$scCO_2$ fluid @ 10 mL/min, 1 h, 40 °C, 20 Mpa with MeOH @ 5 mL/min (entrainer)	10 mg/mL DAN in 70% MeOH + 0.1% TFA	SolariX XR (Bruker)	CsI clusters, Peptide calibration standard, Protein calibration standard I (Bruker)		Wash reduced lipids
[13] $^{\Psi}$	Istrian dry-cured ham	100% IPA @ 60 s, 70% IPA @ 60 s, Carnoy's solution (C.s.) @ 120 s, 70% IPA @ 60 s, *modified C.s.@ 120 s, 100% IPA @ 60 s, ultrapure water @ 240 s, 70% IPA @ 60 s, 100% IPA @ 60 s	25 mg CHCA dissolved in 3 mL of 70/30 acetone/ACN	UltrafleXtreme (Bruker)	Bradykinin fragment 1–5, angiotensin II human, neurotensin, ACTH clip 18–39, insulin bovine β-chain oxidized	ClinProTools v3.0 (Bruker), Mascot Server v.2.6 (Matrix Science, Inc.)	Wash reduced salt and lipids
[16]	Female blue crab brain	50% EtOH @ 10 s	40 mg/mL DHB in 50% MeOH + 0.1% FA	MALDI-LTQ-Orbitrap XL (Thermo)		MSiReader (NC State University)	Wash reduced salt and lipids
[17]	Wistar HAN rat pituitary gland	ice-cold $CHCl_3$ @ 30 s, MeOH @ 30 s, EtOH @ 1 min	7 mg/mL CHCA in 50% ACN + 0.2% TFA	PHI nanoTOF II (Physical Electronics)	Peptide calibration standard II (Bruker)	PHI SmartSoft-TOF, PHI TOF-DR (Physical Electronics)	SIMS tandem MS allowed on tissue *de novo* sequencing
[18]	Male Sprague-Dawley rat spinal cord	70% EtOH @ 10 s, 95% EtOH @ 10 s, 95% EtOH @ 10 s	25 mg/mL DHB in 50% MeOH + 15 mM AmAc + 0.3% TFA	Ultraflex II (Bruker)	Peptide calibration standard II (Bruker)	Origin 9.1	Sample preparation optimized for handling spinal cord tissue
[19]	Female rhesus monkey and male Sprague-Dawley rat brain	$CHCl_3$ @ 30 s	A: 25 mg/mL DHB in 50% ACN + 0.2% TFA B: 35 mg/mL DHB in 50% ACN + 0.2% TFA	RapifleX (Bruker) SolariX 7T 2ω (Bruker)		SCiLS Lab v2019b (Bruker), SIMCA v13.0 (Sartorius Stedim Biotech), GraphPad Prism v7.04 (GraphPad)	Optimized matrices for improved NP extraction (matrix A) and improved spatial resolution (matrix B)
[20]	In-house-bred KO mice and homozygous PACAP KO mice intestine	Deparaffinization: two changes of xylene rehydration in IPA and EtOH	10 mg/mL CHCA in 70% ACN + 1% TFA	RapifleX (Bruker)	Peptide calibration standard II (Bruker)	SCiLS Lab v2018b (Bruker)	Sample preparation allowed discovery of peptide deposits from FFPE tissue
[21]	FFPE cockroach retrocerebral complex	Deparaffinization: 100% xylene @ 3 min, fresh xylene @ 2 min, 100% EtOH	50 mg/mL DHB in 50% ACN	LTQ Velos Pro Orbitrap Elite (Thermo)/	Pierce LTQ Velos calibration (Thermo)/red	PEAKS (BSI)/SCiLS Lab v2018b (Bruker)	Optimized deparaffinization method for FFPE tissue

Ref.	Tissue Type	Wash Steps	Matrix	Instrument	Calibration Samples	Analysis Software	Summary of Results
		@ 2 min, fresh EtOH @ 1 min	+ 0.1% TFA	RapifleX (Bruker)	phosphorus monoisotopic cluster peaks		
[30]	Female C57BL/6 J mouse brain		40 mg/mL DHB in 50% MeOH + 0.1% FA	SubAP-MALDI (ng) UHR (MassTech) + QE-HF (Thermo)		ImageQuest (Thermo)	Ion suppression effect was reduced for subAP MALDI analysis
[34]	Blue crab and green crab pericardial organ		5 mg/mL CHCA in 50% ACN	4800 MALDI TOF/TOF MS (Applied Biosystems)		TissueView (Applied Biosystems)	CE fractionation enhanced NP detection
[35]	American cockroach retrocerebral complex	70% EtOH @ 20 s, dry @ 5 s, 100% EtOH @ 20 s	5 mg/mL CHCA in 50% ACN + 2% TFA	RapifleX (Bruker)	Peptide calibration standard II (Bruker)	SCiLS Lab v2018a (Bruker)	Optimized sample preparation enhanced NP coverage

*Modified C.s.: 60 % (v/v) ethanol, 30 % (v/v) methyl-tert-butyl ether, 10 % (v/v) glacial acetic acid. All other abbreviations are listed in manuscript.

ΨThis report investigated endogenous peptides.